THE ESTIMATION OF PEPSIN, TRYPSIN, PAPAIN, AND CATHEPSIN WITH HEMOGLOBIN

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In the hemoglobin method for the estimation of proteinase, denatured hemoglobin is digested under standard conditions, the undigested hemoglobin is precipitated with trichloracetic acid, and the amount of unprecipitated protein split products, which is a measure of the amount of proteinase present, is estimated with the phenol reagent which gives a blue color with tyrosine and tryptophane.

Hemoglobin, unlike casein and gelatin, is a reproducible substrate. Different batches of hemoglobin are digested at the same rate by a given proteinase solution.

Even when peptidase is present in addition to proteinase, the formation of products not precipitable by trichloracetic acid is due so far as is known to proteinase alone.

Hemoglobin methods have been described for pepsin (Anson and Mirsky, 1932), trypsin (Anson and Mirsky, 1933), papain (Anson, 1937 *a*), and cathepsin (Anson, 1937 *b*). Since the methods were first worked out several minor errors have been corrected, the preparation of the hemoglobin substrates has been simplified, and the estimation procedures have been standardized. Rather than point out the numerous changes which have been made I have considered it simpler and more useful to describe completely the procedures as they are now used in this laboratory. To avoid confusion about results already published no radical changes have been made. The pepsin, trypsin, and papain substrates are the same in composition as those originally described. The cathepsin substrate now contains 0.001 M ammonium sulfate which increases the rate of digestion. In the case of the estimation of pepsin the procedure for the estimation

of the products of digestion and the calculation of the activity units have been modified to conform with the procedures used in the estimation of the other proteinases. The original method gave activity values in terms of pepsin units which are 10–15 per cent higher than the present method.

The Preparation of Hemoglobin.—Whipped beef blood is centrifuged 20–30 minutes. The serum and the white corpuscles which form a thin layer on top of the red corpuscles are siphoned off and the red corpuscles are then mixed with an equal volume of cold 1 per cent sodium chloride solution and after centrifugation the supernatant solution is siphoned off again and the corpuscles are either stored frozen or dialyzed immediately and then stored frozen. The corpuscles are largely freed of color producing substances not precipitable by trichloracetic acid by dialysis in Du Pont cellophane tubing of ¾ inches diameter. Cellophane deteriorates on standing, especially the outer layer of the coil which is most exposed to the air. It is necessary to test the tubing for leaks. One end of the tubing is wetted with water and a knot is tied in the end of the tubing. The tube is then filled with water and the open end is twisted and folded over. While the folded part is pressed closed with one hand the tubing is squeezed with the other. The squeezing, in addition to showing up leaks, stretches the tubing and thus excessive stretching and dilution during dialysis are avoided. If the tubing is satisfactory the water is poured out, a marble is put in, the tube is filled with the washed corpuscles and the end of the tubing is closed with a knot in the cellophane itself. The tubes are placed in a tall vessel. Cold tap water is run into the lower part of the vessel at a rate sufficient to cause stirring. Occasionally the tubes are inverted and the hemoglobin solution is thus stirred by the marbles. After 24 hours dialysis the hemoglobin solutions from all the cellophane tubes are mixed and the mixed solution is stored frozen in small aluminum containers or cardboard ice cream containers. It is easily possible to prepare enough dialyzed hemoglobin at one time for thousands of proteinase estimations.

To estimate the concentration of protein in the dialyzed corpuscles a 3–5 gm. sample is weighed out in a porcelain evaporating dish, dried

overnight at 105°C., and the dry weight recorded. The number of grams of protein per cubic centimeter of sample is

$$\frac{\text{Weight of dried protein}}{(\text{Weight of sample} - \text{weight of dried protein}) + 0.73\ \text{weight of dried protein}}$$

When it is not convenient to store the dialyzed hemoglobin frozen it can be stored at room temperature as a dry powder. If the hemoglobin solution is frozen while the drying takes place the hemoglobin remains soluble.

Bacto-hemoglobin of the Difco brand can be used for rough work when the blank is not of importance. It consists of dried washed corpuscles. The other commercial hemoglobins, so far as I know, are prepared from unwashed corpuscles or from whole blood. Some of them give results very different from those obtained with the hemoglobin whose preparation has just been described.

Casein and edestin or egg albumin can be used instead of hemoglobin. Gelatin cannot be used since it is not precipitated by trichloracetic acid.

Phenol Reagent.—To the phenol reagent prepared according to Folin and Ciocalteau (1927) twice its volume of water is added. Whenever the phenol reagent is referred to this diluted reagent is meant.

The Estimation of Pepsin

Preparation of Hemoglobin Substrate.—The dialyzed hemoglobin solution is diluted with water to give a solution containing 2.5 gm. protein per 100 cc. and is centrifuged. The small precipitate is rejected. 1.0 mg. merthiolate (Lilly) per 40 cc. is added as a preservative. Toluol should not be used nor should the amount of merthiolate be increased since larger amounts give a significant color with the phenol reagent. The 2.5 per cent hemoglobin solution is stored at 5°C.

4 cc. of 2.5 per cent hemoglobin solution is added with an automatic pipette to a 175 × 20 mm. test tube. Then 1 cc. of 0.3 N hydrochloric acid is added from an automatic pipette. The final pH is 1.6. The acid substrate solution is stored at 5°C. and used within a day or two

since in some cases the blank increases with time. If the substrate solution is kept for more than an hour before being used, the test tube should be stoppered.

Digestion and Color Development.—Digestion is carried out at 25°C. A convenient holder for the tubes is a piece of wood or bakelite with holes slightly larger than the diameter of the tubes. The tubes float upright in the bath. Enzyme and substrate are brought to the digestion temperature before digestion is begun.

1 ml. of enzyme solution is added to 5 ml. of substrate solution and the two solutions are mixed by whirling the tube. After 10 minutes 10 ml. of 0.3 N trichloracetic acid (estimated by titration) is added, the tube is shaken vigorously, and the suspension is filtered.

A filter paper such as Whatman No. 3 which does not absorb split products must be used. The color value of the split products should be the same whether the protein precipitated by trichloracetic acid is removed by filtration or centrifugation.

To 5 ml. of the digestion filtrate in a 50 cc. Erlenmeyer flask are added 10 ml. of 0.5 N sodium hydroxide and 3 ml. of the phenol reagent. The solution is whirled during the addition of phenol reagent. Since the color formed depends somewhat on the rate at which the phenol reagent is added this rate is standardized by adding the reagent as rapidly as is possible and still have the reagent come out of the burette as drops. The color is read against the standard after 2–10 minutes.

The Standard.—The standard tyrosine solution contains 0.0008 milliequivalents of tyrosine (0.0112 mg. tyrosine nitrogen) in 5 ml. 0.2 N hydrochloric acid, with 0.5 per cent formaldehyde as a preservative. The concentration of tyrosine is determined by the Kjeldahl method. 5 minutes are allowed for the color development after the addition of 10 cc. of 0.5 N sodium hydroxide and 3 cc. of the phenol reagent in the manner already described.

In practice a blue glass is used as a standard instead of the blue solution obtained from 0.0008 milliequivalents tyrosine. To avoid the necessity of finding a blue glass which exactly matches the color of the tyrosine-phenol reagent solution a No. 241 Corning glass filter is placed in or above the eye piece of the colorimeter. In the fairly monochromatic red light transmitted by this filter different blues

look alike. The blue glass standard is calibrated with the tyrosine standard and this calibration is frequently checked.

Blank.—10 cc. of 0.3 N trichloracetic acid is mixed with 5 cc. of hemoglobin solution, 1 cc. of enzyme solution is added, the tube is again shaken, and the suspension is filtered. When a purified enzyme solution is used for digestion the blank is not increased by the enzyme solution and 10 cc. of 0.3 N trichloracetic acid is added to a mixture of 5 cc. hemoglobin solution and 1 cc. water. 1 cc. of a tyrosine solution containing 0.0008 milliequivalents of tyrosine dissolved in 0.1 N hydrochloric acid containing 0.5 per cent formaldehyde is added to 5 cc. of the blank trichloracetic acid filtrate. The color is then developed by the addition of 10 cc. sodium hydroxide and 3 cc. phenol reagent and read after 5 minutes against the standard.

Calculations.—Color value of 5 cc. digestion filtrate in milliequivalents tyrosine =

$$\frac{\text{Colorimeter reading for standard}}{\text{Colorimeter reading for digestion filtrate}} \times 0.0008.$$

Color value of 5 cc. blank filtrate =

$$\left(\frac{\text{Colorimeter reading for standard}}{\text{Colorimeter reading for blank filtrate + added tyrosine}} \times 0.0008 \times \frac{19}{18}\right) - 0.0008$$

Unless split products are added with the enzyme the blank is usually about 0.00008 milliequivalents of tyrosine.

Color value of digestion products in 5 cc. of digestion filtrate = color value of 5 cc. of digestion filtrate − color value of 5 cc. blank filtrate.

The number of activity units corresponding to the color value of the digestion products in 5 cc. of digestion filtrate is read off from the curve (Fig. 1). When the blank is constant one can omit the calculation of color values and use a curve in which the colorimeter reading for 5 cc. digestion filtrate is plotted against activity units.

The Estimation of Trypsin

Hemoglobin Substrate.—A solution is made up containing 8 cc. of 1 N sodium hydroxide, 72 cc. water, 36 gm. urea, and 10 cc. of 22 per cent hemoglobin (22 gm. hemoglobin per 100 cc. solution). This alkaline solution is kept at 25°C. for 30–60 minutes to denature the

hemoglobin and is then mixed with a solution containing 10 cc. 1 M potassium dihydrogen phosphate and 4 gm. of urea. The final pH is 7.5. 1 mg. merthiolate (Lilly) is added to each 50 cc. of hemoglobin solution as a preservative. The hemoglobin solution is stored at 5°C. and is stable for weeks.

Smaller or larger quantities of substrate solution can, of course, be made up so long as the components are added in the manner and the proportions given.

The activity curve is given in Fig. 2.

The procedure for the estimation of trypsin is the same as that for the estimation of pepsin except that because of the urea in the substrate solution it is necessary after the addition of trichloracetic acid to wait 30 minutes before filtration both in the preparation of the digestion filtrate and in the preparation of the blank filtrate.

The Estimation of Papain

The hemoglobin substrate and the estimation procedure are the same as in the estimation of trypsin. The papain must be activated before estimation.[1] This is done as follows: To 0.5 ml. papain solution is added 5 drops 2 N sodium cyanide. After 3 minutes at 25°C. 9.25 ml. of water is added. If further dilution is necessary it is carried out with a solution containing 5 drops of 2 N sodium cyanide in 10 ml. of water.

To obtain the papain activity curve (Fig. 3) digestion was carried out with activated aqueous extract of Optimo Papain (S. B. Penick and Company). The amounts used for digestion corresponded to 0.06–0.3 mg. of the original powder. It is not known whether or not all samples of commercial papain give the same activity curve.

The Estimation of Cathepsin

Preparation of Hemoglobin Substrate.—There is added from an automatic pipette to a 175 × 20 mm. test tube 4 ml. of centrifuged 2.5 per cent hemoglobin solution containing 1 mg. merthiolate per

[1] The activation procedure described gives more rapid and complete activation of crude commercial papain than the usual activation in less alkaline solution. It has not been proved that it gives complete activation. The cyanide is necessary to eliminate inhibitors in the hemoglobin solution as well as to activate the enzyme. The amount of cyanide needed for this purpose may vary with different hemoglobin preparations.

40 cc. (the same solution used for the preparation of the pepsin substrate). From another automatic pipette 1 ml. of a solution 1.35 M in respect to acetic acid and 0.02 M in respect to ammonium sulfate is added. The final pH is 3.5. The acid substrate solution is stored at 5°C. and used within a day or two before the blank increases.

The estimation procedure is the same as that used in the estimation of pepsin except that the digestion is carried out at 37°C. instead of 25°C. The concentration of ammonium sulfate in the enzyme solution should not be greater than 0.04 M (0.01 saturated) since ammonium sulfate in greater concentration decreases the rate of digestion.

Purified beef spleen cathepsin was used to obtain the activity curve (Fig. 4).

Activity Units and the Construction of the Curves

This section defines the activity units and gives the directions for constructing a curve relating activity units to color values of digestion products. It is not necessary to read this section in order to use the procedures for the estimation of pepsin, trypsin, papain, and cathepsin which have been described, since the curves are already given. If the hemoglobin method is applied to other proteinases, however, a new curve must be worked out in each case and, in each case, the proper substrate solution must first be found.

One unit of proteinase is defined as the amount which digests hemoglobin under the standard conditions at an *initial* rate such that there is liberated per minute an amount of split products not precipitated by trichloracetic acid which gives the same color with the phenol reagent as 1 milliequivalent of tyrosine. This unit is similar to the other proteinase units previously used in this laboratory (Northrop, 1932). The specific activity is the activity per milligram of enzyme nitrogen. The standard temperatures are taken to be 25°C. for papain and 37°C. for cathepsin, these being the temperatures used in practice. In the cases of pepsin and trypsin, the first two proteinases studied, although in practice the digestion is carried out at 25°C. the standard temperature was taken to be 35.5°C. that being the standard temperature previously used in this laboratory. The initial rate of digestion by pepsin is 1.82 times greater at 35.5°C. than at 25°C. With trypsin the rate is 1.78 times greater at 35.5°C. than at 25°C.

To obtain an activity curve, the hemoglobin solution is digested for 10 minutes with different amounts of enzyme expressed as cubic centimeters of some stock solution. A curve is plotted relating the color values of the digestion products, *i.e.* the color values of the digestion filtrates corrected for the blanks, to the amounts of enzyme used. A line is drawn tangent to the first part of the curve and there is read off from this line the amount of enzyme which gives a color value of 0.001 milliequivalents of tyrosine for 5 cc. or $\frac{5}{16}$ of the whole trichloracetic acid filtrate after 10 minutes of digestion. This amount of enzyme multiplied by $1000 \times \frac{5}{16} \times 10$ is the one unit of enzyme which produces split products with a color value of 1 milliequivalent of tyrosine in the whole 16 cc. of solution per minute.

The number of activity units per cubic centimeter of stock solution is now known and one can replot the curve to give activity units against color values.

A convenient practical hemoglobin unit which has no theoretical significance or theoretical relation to units previously used is this. When hemoglobin is digested for 10 minutes by 1 practical hemoglobin unit of proteinase at the temperature regularly used the color value of the digestion products in 5 cc. of trichloracetic acid filtrate is 0.0008 milliequivalents of tyrosine. For each proteinase an empirical curve must be obtained relating activity units to color values. It is not necessary to obtain the first part of the curve which is not used in practice and which cannot be obtained as accurately as the middle portion of the curve. The curves given for the standard units can be used to calculate the curves for the practical units.

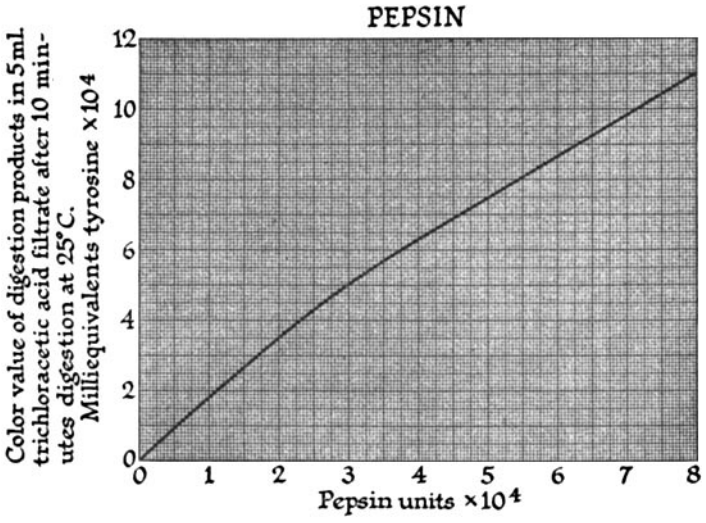
PEPSIN
Color value of digestion products in 5 ml. trichloracetic acid filtrate after 10 minutes digestion at 25°C.
Millequivalents tyrosine × 10⁴
0
2
4
6
8
10
12
0 1 2 3 4 5 6 7 8
Pepsin units × 10⁴

FIG. 1

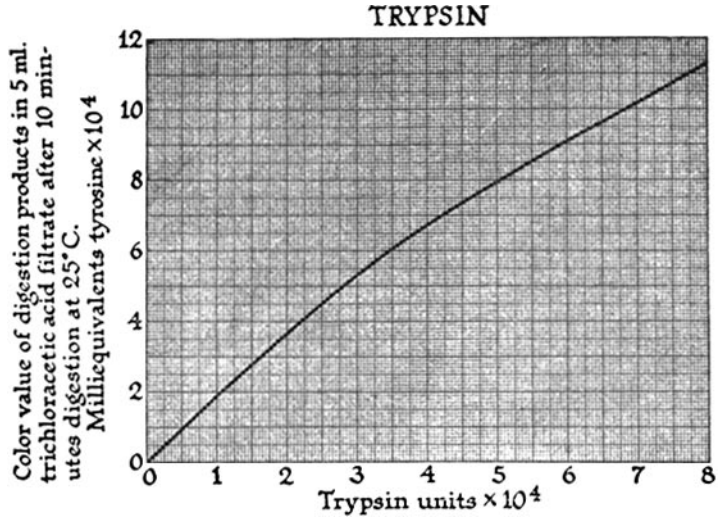
TRYPSIN
Color value of digestion products in 5 ml. trichloracetic acid filtrate after 10 minutes digestion at 25°C.
Millequivalents tyrosine × 10⁴
0
2
4
6
8
10
12
0 1 2 3 4 5 6 7 8
Trypsin units × 10⁴

FIG. 2

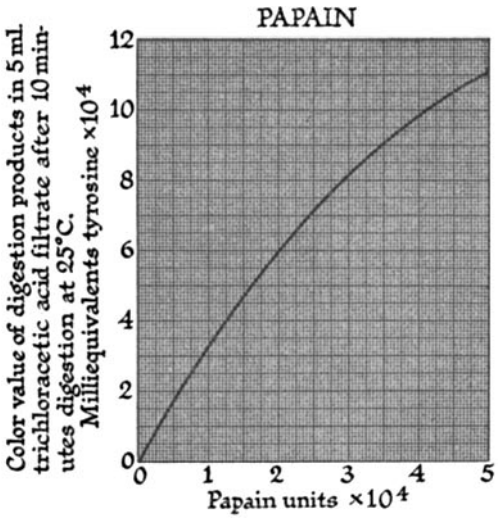
PAPAIN
Color value of digestion products in 5 ml. trichloracetic acid filtrate after 10 minutes digestion at 25°C.
Milliequivalents tyrosine ×10⁴
0
2
4
6
8
10
12
0
1
2
3
4
5
Papain units ×10⁴

FIG. 3

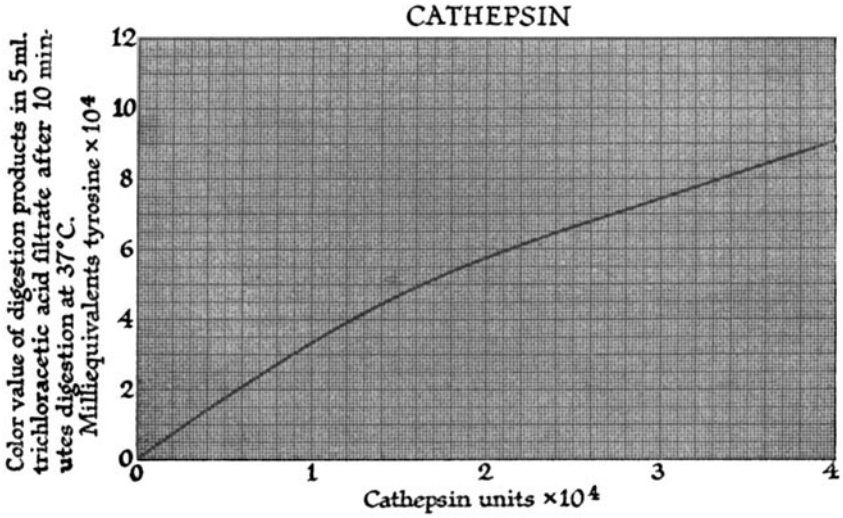
CATHEPSIN
Color value of digestion products in 5 ml. trichloracetic acid filtrate after 10 minutes digestion at 37°C.
Milliequivalents tyrosine × 10⁴
0
2
4
6
8
10
12
0
1
2
3
4
Cathepsin units ×10⁴

FIG. 4

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